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Genotoxicity of Complex Mixtures Strategies for the Identification and Comparative Assessment of Airborne Mutagens and Carcinogens from Combustion Sources (Journal Version)

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Genotoxicity of Complex Mixtures: Strategies for the Identification and Comparative Assessment of Airborne Mutagens and Carcinogens from Combustion Sources[1,2]

JOELLEN LEWTAS

Genetic Bioassay Branch, Genetic Toxicology Division, Health Effects Research Laboratory, Office of Research and Development, U.S. Environmental Protection Agency, Research Triangle Park, North Carolina 27711

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The importance of understanding the toxicology of complex mixtures has been stimulated by the recognition that most human exposure to environmental chemicals occurs as complex mixtures. The toxicology of mixtures has been approached by studying either (1) the individual chemicals in a mixture and their interactions when combined, or (2) the whole mixture or a major component of the mixture (e.g., the particles from a mixture of gases and particles).

The assessment of toxicological interactions between chemicals in simple mixtures is based primarily on studies of two component mixtures of drugs, pesticides, or other well-studied toxic chemicals. The principles proposed for toxicological interactions (NRC, 1980; Murphy, 1980) have been based on the mechanisms elucidated for these simple two-component mixtures generally administered at relatively high doses. These studies have

[1] Presented at the 25th Annual Meeting of the Society of Toxicology, March 1986, New Orleans, Louisiana.

[2] The research described in this paper has been reviewed by the Health Effects Research Laboratory, U.S. Environmental Protection Agency, and approved for publication. Approval does not signify that the contents necessarily reflect the views and policies of the Agency nor does mention of trade names or commercial products constitute endorsement or recommendation for use.

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led to the use of additivity models for chemicals where no interaction data to the contrary are available (USEPA, 1985).

Prediction of the toxicology of complex mixtures based upon component analysis and additivity models, although widely used when no other data is available, presents several major problems recently addressed by a National Research Council report on complex mixtures (NRC, 1988). The most serious problem is that in many cases the toxicologically important components are not detected or quantified because they are present in very low concentrations. Therefore, to assess the toxicology of complex mixtures, it is preferable to conduct studies on the whole mixture or major components of the mixture. The U.S. Environmental Protection Agency in Guidelines for Health Risk Assessment of Chemical Mixtures (USEPA, 1985) also concludes that it is preferable to have health effects data on the mixture of concern or on a similar mixture. The infinite number of possible complex mixtures to which humans are exposed, however, also presents a second serious problem. That problem is the selection of appropriate mixtures to evaluate and the use of data from one mixture to predict the toxicology of another mixture.

This paper presents two strategies for assessing complex mixtures which deal with these problems: (1) the identification of toxicologically important components in complex mixtures, and (2) the comparative assessment of a series of related complex mixtures. Definition of the complex mixture problem and evaluation of any prior knowledge of the mixtures composition, human exposure, and known toxic effects should preceed selection of either of the strategies described here for evaluating complex mixtures.

The strategies described here are only applicable to complex mixtures for which a toxicological effect has been determined. The toxicological methods used to assess whether there are toxic effects from exposure to a mixture are essentially the same as are used for individual chemicals except that special care must be taken in selecting, preparing, and administering the mixture. Once an effect of concern is identified, other strategies may be applied to identify specific toxic components or to assess related mixtures for comparative or predictive purposes.

Combustion products represent one of the major and most widespread sources of complex mixtures to which humans are exposed. This paper describes a strategy for identifying toxic (in this case genotoxic) components in a complex mixture and conducting a comparative assessment of a series of related complex mixtures using the genotoxicity of combustion particle emissions (soot) as an illustration of how these strategies have been applied to increasing our understanding of the potential human risk from exposures to these combustion products.

COMBUSTION EMISSIONS

Combustion emissions are very complex mixtures of thousands of chemicals resulting from incomplete burning of materials derived from vegetation (e.g., tobacco, wood, etc.), fossil fuels (e.g., oil, coal, gasoline, diesel fuel), synthetic sources (e.g., synfuels, other chemical products), or mixtures of these materials (e.g., garbage and waste). The products resulting from incomplete combustion generally contain a complex mixture of organic chemicals including aromatic and aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), polar substituted PAHs and other polar organic compounds. The emission mixture itself is heterogeneous and consists of gases (e.g., CO, NO_x, SO_2, hydrocarbons, and aldehydes), semi-volatile organic compounds (e.g., 2- to 3-ring aromatics), and condensed organic and inorganic matter adsorbed onto small (usually submicron) carbonaceous particles (soot) or other noncarbonaceous particles (e.g., alumina-silica).

These mixtures cannot now or in the forseeable future be completely characterized chemically because they contain thousands

of components. Toxicological studies on the individual components are, therefore, an unsatisfactory means of assessing the toxicology of the total mixture. The mixtures, can, in most cases, be reproducibly generated by the specific emission source (e.g., automobile on a dynamometer) to generate a mixture similar to one to which humans are exposed (Bradow, 1982). Considerable engineering effort has been devoted to standardizing combustion conditions and in some cases even the fuels, so that emissions can be reproducibly generated. This has been done in order to chemically and physically characterize the emissions. These same techniques may be applied to the generation of a representative emission source for toxicological studies.

The toxicological data base on combustion emissions varies from being extensive for some sources such as cigarette smoke, coal soot, and certain automotive emissions (e.g., diesel emissions) to very limited for others, such as, incineration, wood combustion, and synfuels. The more extensive toxicological data available for certain sources, including human data, have made it possible to draw conclusions as to the utility of various methods and approaches which may be generalized to other combustion emissions.

Soot from combustion has, in general, been judged to be carcinogenic in humans (IARC, 1985) and soots from several sources have also been found to be carcinogenic in animals (IARC, 1985). The condensed organics extracted from various combustion soot particles has been extensively evaluated in both mutagenicity and carcinogenicity bioassays (Hoffman and Wynder, 1976; Lewtas, 1985). These organics induce mutations in bacteria and mammalian cells, as well as DNA damage, chromosomal effects, and oncogenic transformations in mammalian cells (Hueper *et al.*, 1962; Claxton and Huisingh, 1980; Lewtas, 1982, 1983, 1985). The carcinogenicity of these organics has been most extensively studied in the mouse skin model (Grimmer et al., 1982; Nesnow *et al.*, 1982a, 1982b); however, they have been shown to induce cancer in other models as well (Hoffman and Wynder, 1976). Until recently, very few lifetime inhalation or intratracheal instillation carcinogenicity studies had been conducted on combustion emissions other than cigarette smoke. Several such studies have now shown that whole diesel emissions, when administered by inhalation, induce a dose-dependent increase in lung carcinomas (Ishinishi *et al.*, 1986). These studies taken together provide strong support to the IARC evaluation (IARC, 1985) that the organics associated with soot are not only human carcinogens, but also are carcinogenic in animals, and mutagenic in bacteria and mammalian cells.

Combustion emissions, therefore, provide a good example of a series of complex mixtures in which an effect of concern, specifically cancer and mutation, have been identified and for which bioassay methods have been established. The two strategies described and illustrated with examples from combustion emission studies are (1) the bioassay-directed fractionation and chemical characterization strategy to identify specific components in a complex mixture which may be the "causative agents" inducing the effect of concern, and (2) the comparative potency method for assessing a series of related complex mixtures for the purpose of predicting either relative human risk or predicting the effect of untested but similar complex mixtures.

BIOASSAY-DIRECTED FRACTIONATION AND CHARACTERIZATION STRATEGY

General Approach to this Strategy

The objective of bioassay-directed chemical characterization is to identify the biologically active (e.g., toxic, mutagenic) components in complex mixtures. This and other approaches to identifying genotoxic compounds in complex mixtures have been re-

viewed by Claxton (1982) and Schuetzle and Lewtas (1986). The two principle considerations in applying this approach are the bioassay to be employed and the fractionation method, both of which are discussed below.

The bioassay(s) chosen will depend on toxicological effects observed for the whole mixture or the components. Often the source of the mixture and what is known about either the chemical composition or toxicological effects of similar mixtures will suggest a particular effect. Examples of bioassays which have been used include assays for mutagenicity (e.g., Ames test), tumor initiation, cytotoxicity, etc.

The methods which have been used to fractionate complex mixtures prior to bioassay include nearly all the methods which have been used for separations prior to chemical analysis, and often several of these methods are combined sequentially. One of the most widely used approaches to mixture separation is solvent partitioning into organics and inorganics. These will each be bioassayed. If most of the activity of interest is in the organic fraction, it will be further solvent-partitioned into organic acids, organic bases, and neutral compounds. If bioassay of these three fractions shows that the activity is largely isolated into the organic neutral fraction, it may be further fractionated by silica gel chromatography or high-pressure liquid chromatography (HPLC) into the aliphatics, aromatics, and moderately polar and highly polar neutral compounds. This reiterative approach, diagrammed in Fig. 1, is used repeatedly until the active compounds have been separated from the nonactive components and the active fraction is simplified to allow chemical characterization of the biologically active compounds.

At each point in the fractionation scheme where activity is measured, recovery of mass and activity is also determined. A reconstituted mixture is bioassayed and compared to the unfractionated (neat) mixture to determine the recovery of bioactivity. If the sum of the activity of the mass-weighted fractions

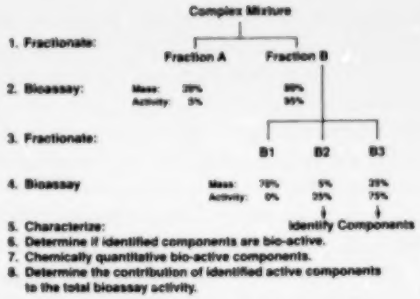

FIG. 1. General schematic diagram of bioassay-directed fractionation and characterization.

equals the unfractionated (neat) mixture than the toxicity is often considered to be additive. Chemical changes resulting from the fractionation procedure could both increase the activity of some components and decrease the activity of others, resulting in the appearance of no change and an incorrect conclusion that biological activity is recovered.

In several cases this approach has resulted in the identification of biologically active components of mixtures which were very minor components by mass but the major components responsible for the mixture's biological activity due to the high potency (biological activity per unit mass) of the compound. Examples of such highly potent components are the mutagenic dinitropyrenes found in xerographic toners (Rosenkranz *et al.*, 1980) and diesel exhaust (Nishioka *et al.*, 1982; Schuetzle, 1983); and aflatoxin, found in peanuts (NRC, 1988).

Once a highly active fraction is obtained and chemical analysis provides a list of the confirmed or suspected components, one critical requirement must be met before biologically active compounds are identified. Either bioassay data must be available for each of the compounds identified, and/or pure standards need to be obtained to bioassay the identified compounds in order to determine their contribution to the total activity observed.

The first studies conducted in the 1920s which employed this general approach ap-

plied coal tar fractions to animals to identify the fluorescent carcinogenic components (Dipple, 1976). Although these studies did not quantify the biological response as described above, they were successful in identifying polycyclic aromatic hydrocarbons in general and benzo[*a*]pyrene in particular as important carcinogens in these complex mixtures. Hoffman and Wynder (1976) review these early studies and the studies that followed which identified other important PAH. The examples that follow, illustrate recent developments in applying and improving this general strategy in order to identify the genotoxic components in combustion emissions and ambient air.

Characterization and Identification of the Mutagens and Carcinogens Emitted from Combustion Sources

Bioassay-directed fractionation closely coupled to chemical characterization has been recently reviewed by Schuetzle and Lewtas (1986) and by the NRC (1988), who also compared this method to other approaches described by Claxton (1982). Bioassay-directed chemical characterization is a more efficient and effective approach to identifying biologically active compounds. This approach has been used to identify tumor initiators and tumor promoters in cigarette-smoke condensates (Hoffman and Wynder, 1976), automotive exhaust emissions (Grimmer *et al.*, 1982), and urban-air particles (Hueper *et al.*, 1962). This approach has also been coupled with short-term genetic bioassays, including both microbial and mammalian-cell mutation assays, to identify mutagens and potential carcinogens in complex mixtures (Epler *et al.*, 1978). We first employed this method to identify the chemical classes and specific components associated with diesel particulate emissions that were mutagenic in the Ames *Salmonella typhimurium* mutagenesis assay (Huisingh *et al.*, 1979).

Diesel particles from a heavy duty engine collected on filters after dilution were extracted with dichloromethane and solvent-partitioned into organic acids, bases, and neutral components. The neutral components were further fractionated on a silica gel column into paraffins, aromatics, and moderately polar and highly polar fractions. The mutagenic activity of each fraction was determined using the Ames *Salmonella typhimurium*/microsome assay (Huisingh *et al.*, 1979). A major portion (65%) of the mutagenic activity in TA98 in the absence of S9 was in the moderately polar fraction which contained only 5% of the mass. The moderately and highly polar neutral fractions together accounted for 89–94% of the mutagenic activity (with and without activation in TA98) and only 32% of the mass. Conventional gas chromatography/mass spectroscopy identified many fluorenones and methylated fluorenones as major constituents of those fractions which we found to be nonmutagenic. None of these or other identified constituents accounted for the direct-acting frameshift mutagenic activity observed. Comparative studies with and without nitroreductase-deficient (NR) strains of *Salmonella typhimurium* showed a reduction in the mutagenicity of these organics with the NR strains, suggesting that nitroaromatic compounds contributed to this mutagenicity (Claxton and Huisingh, 1980). Nitrated polycyclic aromatic hydrocarbons (NO_2-PAHs) are potent direct-acting frameshift mutagens detected during this same time in xerographic toners (Rosenkranz *et al.*, 1980). A series of NO_2-PAHs in diesel extracts were later identified and quantitated in order to estimate their contribution to the mutagenic activity of diesel particulate emissions (Nishioka *et al.*, 1982) as described in the next section. Similar studies have also been conducted by Schuetzle (1983) who showed that NO_2-PAHs, di-NO_2-PAHs, and hydroxy-NO_2-PAHs together account for much of the mutagenicity observed in *Salmonella typhimurium*. Particulate emissions from catalyst-

equipped gasoline-engine vehicles using unleaded fuel contain significantly less of these NO_2-PAHs (Nishioka *et al.*, 1982). The mutagenic activity of both leaded- and unleaded-gasoline emissions is substantially increased with the addition of an exogenous metabolic (MA) system, suggesting that the unsubstituted PAHs may play a more important role than do NO_2-PAHs in the mutagenicity and carcinogenicity of gasoline emissions (Lewtas, 1982, 1983).

Characterization of the mutagenicity of emissions from both vegetative and fossil fuels show general similarities. Comparison of the distribution of mutagenic activity in different organic fractions extracted from an airtight woodstove burning oak are compared to the emissions from a light-duty diesel automobile and urban-air particle extracts is summarized in Table 1. Very little mass or mutagenic activity was observed in the organic bases. Urban-air particulate extracts in this and other studies, generally have more mutagenic activity in the acid fraction than primary combustion emissions. This shift in mutagens to the acidic fraction may result from the formation of mutagenic atmospheric transformation products which are highly polar and acidic. Most (78–95%) of the mutagenicity for all these mixtures was in the neutral fraction, however; there were differences in the distribution of mass and mutagenicity in the neutral subfractions.

Bioassay-directed fractionation and chemical characterization has also been used to characterize and compare the complex organic emissions from coal tar used in roofing pots, coke ovens, and cigarette smoke. Fractionation was accomplished by solvent-partitioning the organics into acidic, basic, nonpolar neutrals, aromatics and polar neutrals, and a highly polar fraction (Williams *et al.*, 1986). Characterization of the distribution of mutagenic activity (in *Salmonella typhimurium*) in each of these fractions showed significant differences among the diesel, coke oven, roofing coal tar, and cigarette-smoke condensate samples (Austin *et al.*, 1985). In the diesel samples, over 90% of the mutagenic activity was in the aromatic and polar neutral fractions, and a significant portion of this activity was accounted for by NO_2-PAHs. The other complex organics did not contain detectable amounts of NO_2-PAHs (Williams *et al.*, 1986). Most of the mutagenicity of coke oven sample was found in the basic fraction (37%) and polar neutral fraction (39%). The cigarette-smoke condensate sample also had significant activity in the basic fraction (66%), but chemical analysis indicated that the components differed significantly from those of the coke oven sample. The roofing coal tar sample contained aromatic (14%) and polar (75%) mutagenic constituents that were not NO_2-PAHs. The PAH subfraction of each of these samples accounted for only a small portion of the mutagenicity [e.g., diesel (0.2%), cigarette-smoke condensate (0.1%), roofing tar (5%), and coke oven main (8%)].

Although the specific mutagens in these different sources are not identical, they all cause frameshift mutations and appear to be compounds that could be classified as polycyclic organic matter (POM). Chemical characterization suggests that in addition to nitrated NO_2-PAHs found in the slightly and moderately polar neutrals, hydroxylated and carboxylated polycyclic organics are found in the organic acid fraction, aromatic amines and nitrogen heterocycles are found in the organic bases and highly oxygenated quinones, diones, and nitrooxygenated compounds are found in the polar neutral fractions.

Contribution of NO_2-PAHs to the Mutagenic Activity of Automotive Emissions and Urban-Air Particles

Although NO_2-PAHs were qualitatively identified in extracts of diesel particles and urban-air particulate matter as described earlier, the detection and quantification of these compounds at low levels has posed problems for analytical chemists because the conventional analytical techniques for quantifying

TABLE I

CHARACTERIZATION OF MUTAGENICITY FROM COMBUSTION SOURCES AND URBAN AIR

Fraction[a]	Diesel auto[b]		Wood stove[c]		Urban air[d]	
	Mass (%)	Ames TA98 +S9 mutagenicity (%)[e]	Mass (%)	Ames TA98 +S9 mutagenicity (%)	Mass (%)	Ames TA98 +S9 mutagenicity (%)
1. Organic acids	7.6	17.9	44.0	0.0	7.3	21.0
2. Organic bases	0.2	<0.1	2.2	4.7	0.8	1.0
3. Neutrals						
A. Aliphatics (hexane)	56.1	0.0	14.2	27.9	21.5	0.0
B. Aromatics (hexane/benzene)	12.4	37.0	18.1	23.3	13.6	12.1
C. Moderately polar (dichloromethane)	3.1	24.7	29.0	11.6	8.6	45.0
D. Highly polar (methanol)	20.6	20.4	38.7	32.6	34.0	21.0

[a] The acids and bases were first separated by liquid–liquid partitioning to separate the acids and bases. The neutrals are partitioned into four fractions by open column chromatography on 5% water-deactivated silica gel utilizing the solvents designated in parenthesis.

[b] Diesel particles collected by the dilution tunnel technique from a 1973 Datsun Nissan 220C diesel automobile operated on a chassis dynamometer using the highway fuel economy test cycle (HWFET). The total exhaust was diluted (10:1) with filtered air prior to collection on 20 × 20-in Teflon-coated Pallflex T60-A20 filters. The particles were Soxhlet extracted with dichloromethane (DCM) for 48 hr prior to fractionation.

[c] Wood combustion particles collected by the dilution tunnel technique from an airtight woodstove burning oak (Johnson Energy Converter) during the constant burn phase. The emissions were diluted, collected, and Soxhlet-extracted by the same techniques employed for the diesel particles.

[d] Urban air particles (<1.7 μm) collected by a massive air volume sampler in an industrial/residential area of Philadelphia, PA. The particles were Soxhlet-extracted with DCM for 24 hr prior to fractionation.

[e] Data from the *Salmonella typhimurium* plate incorporation assay in TA98 with metabolic activation was analyzed by the model slope method to determine the specific mutagenic activity in revertants/microgram of fraction tested. Distribution of mutagenic activity between each fraction (% mutagenicity) is the percentage of weighted mutagenic activity [mass (%) × specific mutagenic activity (rev/μg) of each fraction] of each fraction compared to the sum of the weighted mutagenic activities.

PAHs [e.g., high-performance liquid chromatography with fluorescence detection and gas chromatography (GC)/mass spectrometry (MS) with electron impact ionization (EI)] are relatively insensitive to nitro-substituted PAHs. The 1,3-; 1,6-; and 1,8-dinitropyrene isomers are so highly mutagenic in the Ames assay that trace concentrations could account for a major proportion of the observed mutagenic activity of a mixture. A capillary column GC/MS analytical technique using on column injection and negative chemical ionization (NCI) detection was therefore developed to detect these and other nitro-PAHs at very low concentrations (Nishioka *et al.*, 1982). This technique was applied to extracts of soot particles from diesel and gasoline vehicles and urban air particles. Twenty-three different NO_2-PAHs were identified in the diesel engine extracts; five NO_2-PAHs in the urban air particle extracts; and only one, 1-nitropyrene, was detected in the unleaded gasoline-engine extract. 1-Nitropyrene was the NO_2-PAH detected in greatest quantity in the diesel extracts (107–1590 ppm, relative to the weight of the extract), followed by the nitrophenanthrene/anthracene isomers. The only dinitro-PAHs for which analytical standards were available, the dinitropyrene isomers, were detected in one diesel extract sample at sub-ppm concentrations (0.4–0.6 ppm).

Quantification of the concentration of these NO_2-PAHs and determination of their

TABLE 2

CONTRIBUTION OF NO_2-PAHs TO THE MUTAGENIC ACTIVITY OF PARTICLE EXTRACTS IN *Salmonella typhimurium* TA98 (−S9)

		Concentration and contribution to mutagenic activity (%)					
		1-Nitropyrene		Nitrofluoranthene		Dinitropyrene isomers	
Extract sample	Mutagenic activity (rev/μg) TA98 −S9	Conc. (ppm)	Contrib. (%)	Conc. (ppm)	Contrib. (%)	Conc. (ppm)	Contrib. (%)
Diesel auto 1	13.0	1590	11.0	7.0	1.4	—	—
Diesel auto 2	3.9	589	13.0	1.2	0.8	1.6	26.0
Diesel auto 3	3.5	107	2.7	0.9	0.8	—	—
Gasoline auto 4	1.6	2.5	0.1	—	—	—	—
Urban air A	1.8	1.0	0.05	0.8	1.8	—	—
Urban air B	4.4	0.2	0.004	0.4	0.4	—	—

contribution to the mutagenicity in *Salmonella typhimurium* TA 98 (Table 2) shows that the mono-NO_2-PAHs make relatively minor contributions to the mutagenicity of the urban air particles (<2%). 1-Nitropyrene was present at the highest concentration (107–1590 ppm) in the diesel particle extracts, and yet it accounted for only 3–13% of the mutagenicity. In urban-air particle extracts, NO_2-PAHs were quantified at less than 1 ppm and accounted for less than 2% of the mutagenicity. 3-Nitrofluoranthene present at 1 to 7 ppm in the diesel samples accounted for 0.8 to 1.4% of the mutagenicity. Although the isomers 1,3-; 1,6-; and 1,8-dinitropyrene were detected only in one diesel sample at 0.4–0.6 ppm (sum of 1.6 ppm), their mutagenic activity (496,000; 629,000; and 870,000 rev/μg, respectively) was high enough to account for 26% of the mutagenicity of this sample. The total mutagenic activity in *S. typhimurium* TA98 that was accounted for by the 23 nitro-PAHs quantified in Diesel C was 40%. This estimation was supported by the loss of 50% of the mutagenic activity of this extract when it was assayed in a classical nitroreductase-deficient *S. typhimurium* tester strain developed by Rosenkranz *et al.* (1981).

The fact that dinitropyrenes at concentrations below the ppm level can account for nearly one-third of the mutagenic activity suggests that the presence of other highly potent dinitro-PAHs may account for even more. Pederson (1983) has reported that in several light-duty diesel particle extracts, 50 to 90% of the TA98 mutagenicity is lost when the extracts are tested in a *S. typhimurium* tester strain developed to exhibit resistance to dinitropyrenes, TA98/1,8DNP$_6$. Since this strain may show a resistance to other dinitro-PAHs, it is possible that highly potent dinitro-substituted isomers of other parent PAHs present in the particle extracts may also contribute to the mutagenicity of the moderately polar neutral fraction. Although the di-NO_2-PAHs appear to account for more of the mutagenicity than the mono-NO_2-PAHs, the concentration of several of the NO_2-PAHs in a series of extracts of diesel and gasoline exhaust particles were, however, highly correlated with their mutagenicity both in *S. typhimurium* and L5178Y mouse lymphoma cells and skin tumor initiating activity in Sencar mice as described later.

Identification of Hydroxylated Nitro-PAHs in Urban Air

We have recently identified and quantified hydroxy-NO_2-PAHs in both a polar neutral

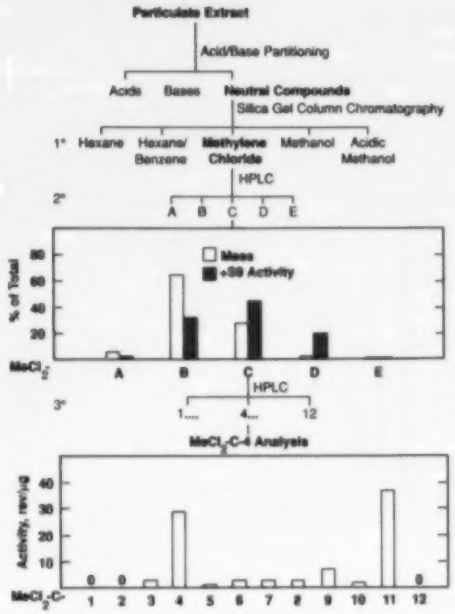

FIG. 2. Bioassay-directed fractionation of urban air particulate.

fraction and acidic fraction of an ambient air particulate extract using new separation and microbioassay methods coupled to the bioassay-directed fractionation strategy (Nishioka *et al.*, in press). Figure 2 shows the overall fractionation and bioassay data. The three fractions of greatest interest were the acid, moderately polar (methylene chloride), and polar (methanol) fractions which contained 38, 23, and 29%, respectively, of the recovered mutagenicity. Normal phase HPLC was used to fractionate the methylene chloride fraction. The first HPLC subfractionation resulted in one fraction, methylene chloride-C, which contained nearly 50% of the mutagenicity in 25% of the mass. Analysis of this fraction by both EI and NCI HRGC/MS techniques indicated that the complexity of the sample was not reduced sufficiently to identify individual components. Subfraction C was further fractionated by normal-phase HPLC into 12 fractions for bioassay. As we proceeded to further subfractionation, less and less mass was available for bioassay. In this study, the first level fractions obtained from the preparative techniques were bioassayed in triplicate at seven doses with and without activation in order to be able to quantitate the distribution and recovery of mutagens. As we proceeded to the first and second level of fractionation, fewer doses and plates were used for the bioassay until nearly the entire fraction was used for a single plate. Therefore, our ability to quantitate the recovery of both mass and mutagenicity decreases as we subfractionate. The bioassay chromatogram shows peaks of relatively higher mutagenicity in fractions 4 and 11 with 29 and 37 rev/μg, respectively. Mass spectral analysis demonstrated that the goal of sequential fractionation has been achieved in that individual components can be resolved and quantified using HRGC/MS. Potentially, highly mutagenic compounds first identified in the methylene chloride-C-4 fraction, and later in the acid fraction, were structural isomers of hydroxylated nitropyrene (e.g., hydroxylated nitrofluoranthenes, acephenanthrylene/aceanthrylene, all MW 202 PAH nuclei). In the methylene chloride-C-11 fraction, a hydroxylated nitroazaarene was identified. Work is continuing to synthesize and identify the specific isomers for bioassay and quantification of their contribution to the total mutagenicity.

Recent Advances in Bioassay-Directed Fractionation: Micro-bioassay Methods Coupled to Analytical Separation Techniques

The quantity of material (mixture or fraction) needed for many of the established bioassay methods ranges from milligram quantities (e.g., Ames assay) to gram quantities (e.g., tumor initiation). Recently a number of micro-bioassay methods have been reported for measuring forward mutation (Thilly *et al.*, 1983; Goto *et al.*, in press), reverse mutation (Kado *et al.*, 1983), in *Salmonella typhimu-*

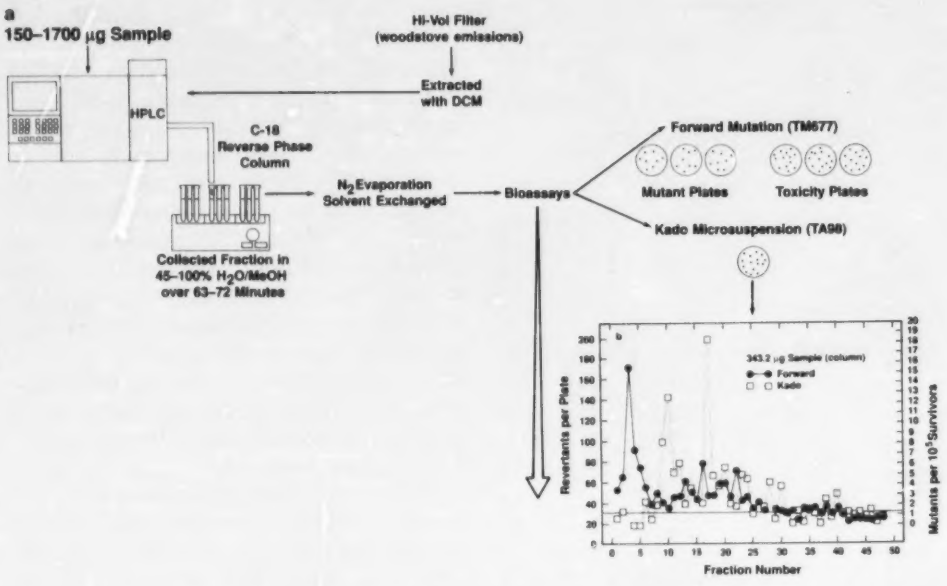

FIG. 3. (a) General schematic of HPLC coupled to microbioassays. (b) Bioassay chromatogram of woodstove emissions using the Forward and Kado micro-mutagenesis bioassays.

rium, and phage induction in *E. coli* (Rossman *et al.*, 1985). Such micro-bioassay methods will facilitate the coupling of bioassay methods to analytical scale separation techniques such as reverse-phase HPLC. Thilly demonstrated the possibilities of this approach by applying the forward mutation micro-assay to particle emission extracts from HPLC fractions of a residential oil burner. Figure 3 demonstrates the utility of this approach when this assay is used to bioassay the fractions from a complex mixture of combustion emissions in ambient air. Such a bioassay chromatogram facilitates the visual identification of the most mutagenic fractions for further characterization. This HPLC/bioassay approach should eliminate the need for large-scale fractionation techniques which are often fraught with problems of the spill-over of chemicals from one fraction to another and with poor resolution. High resolution fractionation techniques used sequentially may make it possible to obtain fractions which contain either one chemical or a very small number of chemicals and thus facilitate characterization of the fractions.

COMPARATIVE POTENCY EVALUATION OF COMPLEX MIXTURES

Complex mixture problems often involve the assessment of a series of different mixtures, such as, fuels derived from various sources, soots emitted from different combustion engines, or air particles from various exposure environments. Comparative studies of a series of substances in one or more bioassays is a useful approach to provide relative toxicity data on different complex mixtures. This general approach has been used in comparing the acute toxicity of a series of petroleum hydrocarbons using a range of products from light oils and gasoline to heavy fuel oils (Beck *et al.*, 1982). The toxicities of shale-de-

rived fuels and other synfuels have been determined primarily in comparative studies in general toxicity tests, target organ studies, behavioral studies, mutagenesis, carcinogenesis, teratology, and neurotoxicity tests. Many of these studies are reviewed by MacFarland *et al.* (1982).

The comparative potency strategy described here focuses on a quantitative comparison of two or more mixtures across at least two bioassays. Potency is used to mean the toxicological response observed per unit of mixture administered in the bioassay. In all of the examples described, the potency is determined from the slope of the initial linear portion of the dose–response curve. The mixtures being compared in these studies do not have the same chemical composition; however, many of these mixtures are very similar (e.g., the series of diesel emissions from different cars) and others (e.g., woodstove emissions and coal tars) all contain polycyclic aromatic compounds which induce frameshift mutations in bacteria and tumors in animals. In addition to the mixtures having similarities, the bioassays are selected to detect endpoints that are thought to be at least mechanistically related; as mutation may induce or be related to tumor initiation, and tumor initiation may preceed and be quantitatively related to tumor progression and final carcinoma incidence.

The design of comparative potency studies requires consideration of factors which may not be as important when evaluating the toxicology of a single chemical or mixture. These factors may include (1) simultaneous evaluation of all mixtures in one experiment to minimize bioassay variability, and (2) exposure doses (or concentrations) needed for statistical analysis and potency determination (Lewtas, 1983; NRC, 1988). The study design should also consider the final use of the data and statistical methods available to maximize future predictions based on the mixture's composition and response-surface modeling (NRC, 1988).

Since all soots from incomplete combustion are expected to contain carcinogenic polycyclic organics, an important public health question is whether a new or alternative combustion source will produce emissions which are more or less carcinogenic than the existing sources. The examples of this strategy which follow are addressing the question of potential human cancer risk from exposures to different combustion emissions.

Comparative Mutagenic and Carcinogenic Potency of Complex Organics from Combustion and Related Sources

Comparison of the potencies of chemical carcinogens, in animals with the mutagenic potency of these chemicals in the Ames *Salmonella typhimurium* assay, was first reported by Meselson and Russel (1977). They observed that there was a potency range of six orders of magnitude between the weakest detectible mutagen or carcinogen and the most potent. They also suggested that there appeared to be a high correlation between mutagenic and carcinogenic potency for these chemicals. As more chemicals have been tested in both the rodent cancer bioassays and the Ames assay, it has become apparent that there are classes of chemicals for which there is a relatively low correlation between these assays and other classes where the correlation is relatively high as shown in Fig. 4 (Sugimura and Takayama, 1983). The correlation between these assays is certainly dependent upon whether a mutagenic mechanism is involved with the induction of cancer for that class of chemicals.

We have evaluated both the mutagenic potency and tumor initiating potency of a wide range of complex mixtures of organics either extracted from combustion soot particles or condensed organics from related products. Both the mutagenic and tumorigenic potency range for these mixtures is less than three orders of magnitude (Lewtas, 1985) compared to at least six orders of magnitude for pure

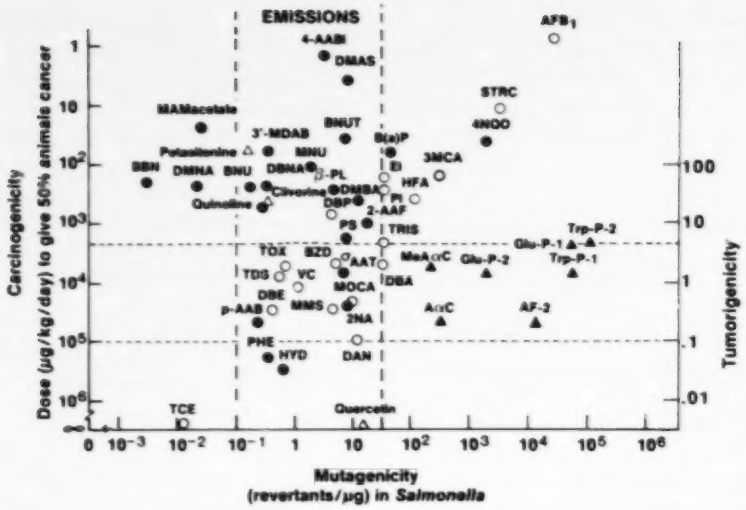

FIG. 4. Comparative carcinogenic and mutagenic potency of pure compounds and complex combustion emissions. Tumorigenicity in Sencar mice is shown as papillomas/mouse/mg. Potency of combustion emission particle extracts in tumorigenicity and mutagenicity assays is shown within the dashed lines.

chemicals. These mixtures fall in the middle of the potency scale for both mutagenicity and carcinogenicity as shown in Fig. 4.

The mutagenicity of the extractable organic material from diesel and gasoline emissions was evaluated for a battery of *in vitro* bioassays detecting gene mutations, DNA damage, chromosomal effects, and oncogenic transformations (Lewtas, 1983). Skin tumor initiation and carcinogenic activity of these samples has also been reported in SENCAR mice (Nesnow *et al.*, 1982). Very good correlations ($r^2 > 0.90$) were observed when the slope of the dose–response for the mutagenic activity in *S. typhimurium* strain TA98 was plotted versus the mutagenic activity in the mammalian cell assays and the skin tumor initiating activity.

The correlation of mutagenic and skin tumor initiating activity with the concentration of selected nitro-PAHs was examined for these diesel and gasoline samples. Table 3 shows the high correlations observed between the concentrations of 1-nitropyrene and 3-nitrofluoranthene in these samples, and their mutagenic activity in *S. typhimurium* (−S9), L5178Y mouse lymphoma cells (−S9), and skin tumor initiating activity in SENCAR mice. The r^2 correlation coefficient in the presence of S9 (not shown) was slightly lower. The mutagenicity and tumor initiation activity (−S9) also correlated well ($r^2 \geq 0.90$) with the concentrations of the nitro-252 isomer [e.g., B(e)P, B(a)P, perylene, benzofluoranthene, etc.], the nitro-228 isomers [e.g., benz[*a*]anthracene, chrysene, benz[*c*]phenanthrene, etc.], nitromethylpyrenes, and nitrofluorenes; while no correlation was observed with several of the other less mutagenic, lower molecular weight nitro-PAHs (e.g., nitrophenanthrenes and nitronaphthalenes).

The quantified mono-NO_2-PAHs account for less than 20% of the direct-acting bacterial mutagenicity of these samples. The high correlations observed between the concentration of these compounds and the mutagenic activity of the total extract (Fig. 5), therefore, suggest that the concentrations of the unidentified mutagens responsible for the remainder of the mutagenic activity and possibly the mutagenic and carcinogenic activity in other

TABLE 3

CORRELATION ANALYSIS OF NITROPYRENE AND NITROFLUORANTHENE CONCENTRATIONS WITH MUTAGENIC AND TUMORIGENIC ACTIVITY

Particle extract	1-NP[a] (ppm)	3-NF[b] (ppm)	Ames TA98 −S9 (rev/μg)[c]	Mouse lymphoma −S9 (MF/μg/ml)[d]	Tumor initiation (pap/mouse/μg)[e]
Diesel auto 1	1590	7.0	13.0	4.2	590
Diesel auto 2	589	2.9	3.9	0.98	240
Diesel auto 3	107	1.2	3.5	1.2	310
Gasoline auto 4	2.5	0.9	1.6	0.38	170
Correlation Coefficient					
r^2 with 1-NP			0.91	0.98	0.82
r^2 with 3-NF			>0.99	0.99	0.95

[a] 1-Nitropyrene.
[b] 3-Nitrofluoranthene.
[c] Revertants per microgram.
[d] Mutation frequency (mutants per 10^6 survivors) per microgram per milliliter.
[e] Papillomas per mouse per microgram in SENCAR mice.

bioassays are directly related to the relative concentrations of these mono-NO_2-PAHs. Because the remaining unidentified mutagens appear to be primarily located in the chemical fractions that are more polar than the fraction which contains the unsubstituted and mono-NO_2-PAHs, it is possible that other di-NO_2-PAHs (e.g., dinitrofluoranthenes) or other oxygenated NO_2-PAHs (e.g., hydroxynitro-PAHs) species are primarily responsible for the major portion of the unidentified mutagenic activity.

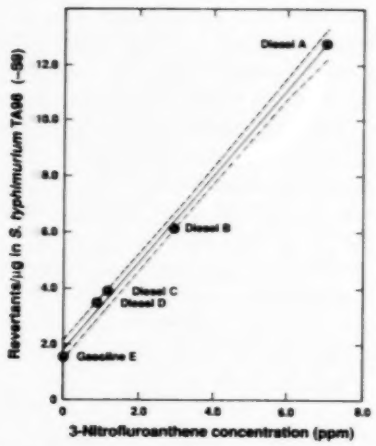

FIG. 5. Correlation of the mutagenicity of automotive emissions with 3-nitrofluoranthene concentration.

Constant Relative Potency Hypothesis: Evaluation of the Underlying Assumption of the Comparative Potency Method

A comparative potency method for cancer risk assessment has been developed based upon a constant relative potency hypothesis. This method was developed and tested using data from a battery of short-term mutagenesis bioassays, animal tumorigenicity data, and human lung cancer risk estimations (Lewtas, 1983; Nesnow *et al.*, 1982a,b; Albert *et al.*, 1983; Lewtas *et al.*, 1981). The data base presented in these papers was developed for a series of complex mixtures including emissions from coke ovens, roofing tar pots, cigarette smoke, and automotive engines.

The comparative potency method for cancer risk assessment is based on the hypothesis that there is a constant relative potency be-

tween two different carcinogens (potency of carcinogen$_1$/potency of carcinogen$_2$) across different bioassay systems (B_1 and B_2). The mathematical expression for the constant relative potency model is the following:

$$\text{Relative Potency in Bioassay}_{(1)} = (k)\text{Relative Potency in Bioassay}_{(2)}$$

The constant relative potency assumption is implicit in any comparison which utilizes the relative toxicity of two substances in animals to determine their relative toxicity in man. This constant relative potency assumption is a testable hypothesis, if the relative potency of two mixtures or components in one bioassay (e.g., humans) can be determined and compared to the relative potency in a second bioassay. The test of this model is whether there is a constant relationship (k) between the relative potencies in the two bioassays being compared such that

$$\frac{\text{Relative Potency in Bioassay}_{(1)}}{\text{Relative Potency in Bioassay}_{(2)}} = \text{constant } (k).$$

This hypothesis was tested for three complex organic emissions from a coke oven, roofing tar pot, and cigarettes by using human lung cancer data from epidemiological studies of humans exposed to these emissions and testing these emissions in a series of short-term mutagenesis bioassays and animal tumorigenesis assays (Albert *et al.*, 1983; Lewtas, 1985).

The validity of this constant relative potency hypothesis for chemical mixtures may depend on the chemical nature of the mixtures being compared as well as the similarity of those mixtures. Since the high correlation between mutagenic and tumorigenic potency in the diesel emissions is also highly correlated with the concentration of nitrated PAH in these mixtures, the validity of the constant relative potency hypothesis, in this case, may be due to the chemical similarity of these diesel emission mixtures. If technological changes do not drastically alter the chemical nature of diesel emissions, but only alter the relative concentrations of those chemicals responsible for inducing mutations and tumors, then it should be possible to predict the tumorigenicity of new diesel technologies by using an Ames bioassay and the comparative potency method.

Application of the Comparative Potency Method to Cancer Risk Assessment

The application of the comparative potency method to cancer risk assessment is dependent upon the human cancer data which may be used for the comparative assessment. The first application in which we have estimated the human cancer risk of a series of combustion emissions from diesel vehicles was based on comparing the bioassay potency of these emissions to three emissions for which human lung cancer data was available: coke oven emissions, roofing coal tar emissions, and cigarette smoke (Albert *et al.*, 1983). In this study, comparative mouse skin tumor initiation data was used together with the human data to estimate the lung cancer unit risk for the most potent diesel emission. Since we found a high correlation between the tumorigenicity and mutagenicity of the series of diesel emissions being evaluated, the mutagenicity data was used to estimate the cancer risk to the other diesels. The average unit lifetime lung cancer risk for three diesel vehicles was 2.6×10^{-5} risk/$\mu g/m^3$ particulate matter. Recently, several lifetime rat inhalation studies of diesel emissions have resulted in dose-related increases in lung tumors which include both pulmonary adenomas and adenocarcinomas (Ishinishi *et al.*, 1986). Albert and Chen (1986), using the rat inhalation data from Lovelace Inhalation Toxicology Research Institute (Mauderly *et al.*, 1986) and combining pulmonary adenomas and adenocarcinoma, calculated the unit lifetime lung cancer risk to be 1.2×10^{-5} risk/$\mu g/m^3$ particulate matter. Considering the

differences in diesel vehicles and assumptions in these cancer risk assessment methods, the difference of only twofold in the unit risk when determined by these two independent methods (which employ different models and assumptions) is remarkable.

The application of the comparative potency method to cancer risk assessment will certainly be limited by the amount and quality of the human data used in the comparative calculations. In order to test the constant relative potency hypothesis across more human data and to potentially extend the application of this method, a comparative assessment of human lung cancer risks, mutagenicity, and carcinogenicity of three exposures (smoky coal, smokeless coal, and wood combustion) is being conducted in Xuan Wei, China (Mumford *et al.*, 1987). Initial evaluation of the mutagenicity and carcinogenicity of these soots, compared to the lung cancer mortality, shows that the correlations between these measurements will be relatively high (Mumford *et al.*, 1987).

Typical unit risk estimates (lifetime risk/$\mu g/m^3$) for commonly encountered combustion sources have been calculated, using the comparative mutagenicity and tumor initiation potency of the complex organics extracted from soot particles emitted from these sources (Table 4). These estimates show only a 150-fold difference in cancer unit risks for these various soot particles from different combustion sources, which range from 0.2 to 30×10^{-5} lifetime risk/microgram particles/cubic meter of air. The amount of extractable organics from various combustion source particles can vary from nearly 100% for woodstove emissions to less than 0.1% for very efficient coal fired power plants. The particle emission rate also varies over many orders of magnitude for various combustion sources.

Another approach to cancer risk assessment of combustion sources, which employs the comparative potency method, is to estimate the relative human risk (rather than the absolute unit risk) by comparing mutagenic and tumorigenic emission rates of soot from various sources. Dose–response studies have been conducted on the extractable organics from these sources in both Ames mutagenicity bioassays and SENCAR mouse skin tumor initiation assays. The slope of the initial linear portion of the dose response is used to calculate the revertants per microgram of organic matter and the papillomas per mouse per milligram of organic matter. These potencies (slopes) are combined with the percentage organic extractable matter and particle emission rate to determine the mutagenic and tumorigenic emission rates for these sources. Examples of typical genotoxic emission rates are shown in Table 5. This analysis shows, more clearly than the method of using a unit risk comparison, the differences between these alternative combustion sources, because an exposure parameter (particle emission rate) has been combined with the potency. This approach to comparing combustion sources provides a useful tool to engineers and public health officials to assess potential relative hazard of sources while avoiding the uncertainties in utilizing human epidemiological data to determine (by comparison) an absolute unit risk which is used

TABLE 4

CANCER UNIT-RISK ESTIMATES FOR PARTICLE EMISSIONS FROM TYPICAL COMBUSTION SOURCES DETERMINED FROM THE COMPARATIVE POTENCY METHOD

Emission source	Unit-risk estimates (lifetime risk/μg particulate matter/m^3)
Automobiles	
Gasoline-catalyst	5.1×10^{-5}
Gasoline-noncatalyst	1.6×10^{-5}
Diesel	3.0×10^{-5}
Trucks	
Heavy-duty diesel	0.2×10^{-5}
Light-duty gasoline-noncatalyst (van)	30.0×10^{-5}
Residential heating	
Oil	0.9×10^{-5}
Wood	1.0×10^{-5}

TABLE 5

TYPICAL MUTAGENIC AND TUMORIGENIC EMISSION FACTORS FROM VARIOUS COMBUSTION SOURCES

Source	Mutagenic emission factors		Tumorigenic emission factors (Papillomas/mouse/kg fuel)
	Revertants/kg fuel $\times 10^5$	rev/MJ or rev/km	
Residential heating			
Wood	50	250,000 rev/MJ[a]	83
Oil	1	2,500 rev/MJ	3
Industrial and utility boilers and power plants			
Oil	0.03	70 rev/MJ	—[c]
Coal	0.06	230 rev/MJ	—
Wood	0.20	1000 rev/MJ	—
Automobiles and trucks			
Diesel vehicles	40	300×10^3 rev/km[b]	240
Diesel trucks/buses	40	1600×10^3 rev/km	—
Gasoline-noncatalyst	10	100×10^3 rev/km	20
Gasoline-catalyst	1	10×10^3 rev/km	2

[a] Revertants per megajoule of heat.
[b] Revertants per kilometer driven.
[c] No data available.

in estimating excess deaths. The assumption, however, that a source with a higher mutagenic or tumorigenic emission rate has a higher human cancer risk is dependent upon the validity of the constant relative potency hypothesis.

Total cancer risk may be underestimated by using only the soot particle extracts in comparative bioassay studies. Combustion sources, as described earlier, emit gases and vapors which do not condense onto the particles and are also known to contain aromatic hydrocarbons, aldehydes, alkenes, and other potentially carcinogenic compounds. Rodent inhalation and human epidemiological studies, which have been discussed above, would include both the soot and gaseous emissions. More research is needed to understand the relationship between the emissions, potential atmospheric transformations, human exposure, dosimetry, and final cancer risk.

SUMMARY

Toxicology studies of complex mixtures employ the same general methods, models, and bioassays that have been developed and validated using single agents. The strategies for combining and applying these methods, however, may differ due to the complexity of the mixture and the questions being addressed. In initial studies to determine the toxicological effects of an unknown mixture, the mixture is administered as a single agent would be in standard *in vivo* or *in vitro* toxicological bioassays. Prediction of the toxicology of a complex mixture from an analysis of its components is generally successful only if the number of components is limited (two to five components) and well defined.

In approaching a very complex mixture where the toxicological effects are known, the two strategies described here can be very useful in identifying toxic components and providing a comparative assessment of similar mixtures. These two strategies have been successfully applied to the evaluation of divergent complex mixtures which cause a variety of effects. These strategies illustrated with air pollution examples are equally applicable to water pollution or hazardous waste prob-

lems. Wastewaters which are acutely toxic to fish have been comparatively evaluated to determine which wastewaters, industrial effluents, or sites are the most toxic using acute toxicity assays in fish (Merrill, 1983). Bioassay-directed fractionation techniques have also been used to identify the class of compounds (e.g., organic vs. inorganic, etc.) responsible for this toxicity. Recent inhalation studies of unleaded gasoline have demonstrated renal toxicity and carcinomas in rodents. Studies of the renal toxicity of different fractions of the gasoline led to the identification of isoparaffins as the active agents (Mehlman *et al.*, 1984).

It is important to keep in mind that the two strategies described here are limited by the bioassay methods employed. If, for example, you wish to identify carcinogens in a complex mixture which is known to contain chlorinated hydrocarbons, then the Ames *Salmonella typhimurium* bioassay would not be a wise choice, since it is well documented that this bioassay does not detect many carcinogens of this class. Likewise, a tumor promoter bioassay based on a receptor binding mechanism would not be likely to detect the most important genotoxic tumor initiators in a mixture. In some cases several bioassay methods may have to be applied in using these strategies to comparatively evaluate mixtures and elucidate the active toxicological agents.

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